Inhibition of Matrix Metalloproteinase Activity by TIMP-1 Gene Transfer Effectively Treats Ischemic Cardiomyopathy

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Background—Enhanced activity of matrix metalloproteinases (MMPs) has been associated with extracellular matrix degradation and ischemic heart failure in animal models and human patients. This study evaluated the effects of MMP inhibition by gene transfer of TIMP-1 in a rat model of ischemic cardiomyopathy.

Methods and Results—Rats underwent ligation of the left anterior descending coronary artery with direct intramyocardial injection of replication-deficient adenovirus encoding TIMP-1 (n = 8) or null virus as control vector (n = 8), and animals were analyzed after 6 weeks. Both systolic and diastolic cardiac function was significantly preserved in the TIMP-1 group compared with control animals (maximum left ventricular [LV] pressure: TIMP-1 70±10 versus control 56±12 mmHg, P<0.05; maximum dP/dt 2697±842 versus 1622±527 mmHg/sec, P<0.01; minimum dP/dt −2900±917 versus −1195±593, P<0.001). Ventricular geometry was significantly preserved in the TIMP-1 group (LV diameter 13.0±0.7 versus control 14.4±0.4 mm, P<0.001; border-zone wall thickness 1.59±0.11 versus control 1.28±0.19 mm, P<0.05), and this was associated with a reduction in myocardial fibrosis (2.36±0.87 versus control 3.89±1.79 μg hydroxyproline/mg tissue, P<0.05). MMP activity was reduced in the TIMP-1 animals (1.5±0.9 versus control 43.1±14.9 ng of MMP-1 activity, P<0.05).

Conclusions—TIMP-1 gene transfer inhibits MMP activity and preserves cardiac function and geometry in ischemic cardiomyopathy. The reduction in myocardial fibrosis may be primarily responsible for the improved diastolic function in treated animals. TIMP-1 overexpression is a promising therapeutic target for continued investigation. (Circulation. 2004;110[suppl II]:II-180–II-186.)

Key Words: gene therapy ■ heart failure ■ metalloproteinases ■ remodeling

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endoproteinases that promote extracellular matrix (ECM) degradation.1 MMPs are produced by fibroblasts, inflammatory cells, and cardiomyocytes in the heart.2 Under ambient conditions, MMP activity is tightly controlled by the endogenous tissue inhibitors of metalloproteinases (TIMPs), of which there are 4 known family members with significant structural homology. The primary physiological role of TIMPs is thought to be regulation and maintenance of ECM homeostasis and integrity. In addition, it appears that TIMPs may play a role in cell growth, apoptosis, and angiogenesis.

Heart failure in human patients and animal models is accompanied by increased MMP activity and decreased levels of TIMPs.3,4 A reduction in myocardial TIMP-1, in particular, is significantly associated with ischemic heart failure in human patients5 and in animal models.6 An imbalance between TIMP and MMP expression appears to be responsible for the increased MMP activity observed in congestive heart failure, which is associated with myocardial matrix collagen disruption and ventricular remodeling.6,7,8 Disruption of the ECM impairs myocardial contractile function and promotes ventricular dilation and wall thinning. Destabilization of intercellular connections promotes cell–cell slippage, an important contributor to ventricular wall thinning.9 In animal models of diverse pathologic processes, pharmacologic inhibition of MMPs attenuated acute pancreatitis,10 improved the strength of intestinal anastomoses,11 decreased retinal neovascularization,12 and inhibited ventricular remodeling.13,14 However, systemic MMP inhibition was associated with severe musculoskeletal pain and tendonitis in clinical trials in patients with metastatic cancer,15,16 necessitating treatment withdrawal in some cases. Systemic inhibition of MMP activity needs further investigation to demonstrate clinical viability without significant side effects.

We hypothesized that myocardial overexpression of TIMP-1 after myocardial infarction (MI) may prevent excessive ECM degradation, and thereby attenuate the deleterious...
remodeling process and functional decline seen in ischemic heart failure. Using a rat model of postinfarction heart failure, we used an adenoviral vector to locally overexpress TIMP-1 in border-zone areas of myocardium and assessed the effects on cardiac function, ventricular geometry, myocardial fibrosis, and MMP activity.

**Methods**

### Adenoviral Vector Construction

Replication-deficient (E1, E3 deleted) adenoviral vectors containing human TIMP-1 (Adeno.TIMP-1) and empty replication-deficient adenovirus containing no transgene as a control vector (Adeno.Null) were both amplified by the University of Iowa Gene Transfer Vector core (supported in part by the National Institutes of Health and the Roy J. Carver Foundation). The DNA construct of replication-deficient Adeno.TIMP-1 comprised an almost full-length copy of the adenoviral genome, into which was cloned the 650-bp coding region of TIMP-1 cDNA17 expression cassette with the Rous sarcoma virus promoter to drive transcription of TIMP-1 preceding the cDNA. A polyadenylation sequence of SV40 was cloned downstream of TIMP-1.18 Purified and concentrated adenoviral vectors (serotype 5), produced in 293 cells and containing ~2×10⁹ pfu/mL, were used in this study.

### In Vivo Transgene Expression

TIMP-1 in vivo viral transgene expression was confirmed by immunoblotting performed after direct intramyocardial injection of either Adeno.TIMP-1 or Adeno.Null control virus (n=3 each) using the methodology described. The hearts were procured after 1 week, myocardial biopsy specimens were normalized for total protein content, and 50 μg of each sample were then electrophoresed on a 12.5% SDS-polyacrylamide gel, and immunoblotting was performed using a rabbit anti-human TIMP-1 polyclonal antibody (Affinity BioReagents, Golden, Colo).

### Animal Surgery

All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (National Academy Press, Washington, DC, 1996) and in accordance with the animal care and use guidelines of the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Twenty-six male Lewis inbred rats (250 to 300 grams, Charles River Laboratories, Wilmington, Mass) were used in the main portion of this study. Lewis rats were chosen because of a consistent infarct size of ~35% of the left ventricle and low mortality after ligation of the left anterior descending coronary artery (LAD).19

Rats were anesthetized with intraperitoneal doses of ketamine (50 mg/kg) and xylazine (5 mg/kg), intubated, and mechanically ventilated with 0.5% isoflurane. A left thoracotomy was performed and the proximal LAD artery was encircled with a 7-0 polypropylene suture. Animals were then randomized to a blinded fashion to 1 of 3 experimental groups: sham, Adeno.Null control (control), or Adeno.TIMP-1 (TIMP-1). In the sham animals (n=8), the suture was removed without tying and no infarction was created. In the control (n=9, 8 survivors) and TIMP-1 (n=9, 8 survivors) animals, the suture was tied to create a large anterior left ventricular infarct that was confirmed by visible blanching of the region at the time of ligation. The control and TIMP-1 animals then received direct intramyocardial injections into the left ventricular (LV) muscular wall of 5×10⁸ pfu of virus into 5 separate locations in the infarction border-zone areas. A total of 250 μL of virus solution was delivered, and injections were performed in a blinded fashion by a single investigator. The total perioperative mortality in the infarcted animals was 2/18 (11%), and both animals died within 24 hours of the initial surgery. There were no late deaths in the control or TIMP-1 animals, and there were no deaths in the sham group.

### Hemodynamic Measurements

After 6 weeks, in vivo hemodynamic measurements were obtained with a 2-French pressure–volume conductance microcatheter (Millar Instruments). In all animals, hemodynamic measurements were obtained at steady state, wherein preload-adjusted maximal power measurements were derived by obtaining hemodynamic measurements with inferior vena cava occlusion to alter preload. All animals were fully anesthetized with intraperitoneal ketamine/xylazine and received inhaled isoflurane on a ventilator. The catheter was placed through the apex of the heart and measurements were performed with the chest open. The heart was arrested in diastole by injection of 0.1 mL of KCl (1 mEq/mL), the LV cavity was filled with optimal cutting temperature (OCT) embedding compound (Sakura Finetek) fixative through the aortic root and stored in a −80°C freezer.

### Ventricular Geometry and Infarct Size

Hearts were obtained from storage and 10-μm sections were prepared with a cryostat and stained with hematoxylin and eosin. Measurements of ventricular geometry were performed on sections obtained from the midportion between the LAD ligation and the apex of the heart. To standardize the geometry measurements, hearts frozen in OCT were sectioned from the aortic root downward toward the apex of the heart until the tie around the LAD was visualized. The midway point between the tie and the apex of the heart (which can be easily identified in frozen OCT) was then identified and the heart was transected at that standardized location. This location was standardized for all animals and was highly reproducible given the consistent areas of infarction in this model and the easily identifiable landmarks of the ligation suture and the apex, as we have previously reported.20 Two sections were obtained from this area, and the diameter and border-zone wall thickness were measured as described in 2 sections and averaged. Measurements were performed on digitized photomicrographs using Openlab image processing software (Improvision) with standards of known length. For all analyses, the border zone was defined as the viable myocardial tissue immediately adjacent to the infarct scar. For the sham animals, measurements were performed on sections obtained from an anatomically equivalent area of the LV. The infarct size in each heart was calculated as the percentage of infarct scar relative to the outer circumference of the LV free wall. A single investigator blinded to the treatment groups performed all measurements.

### Myocardial Fibrosis

A quantitative estimation of collagen content was performed by measuring the hydroxyproline content of border-zone myocardial biopsy specimens. Transmural biopsy specimens weighing ~100 to 200 μg were obtained from the border-zone areas of all animals. Amino acid content analysis was performed by ABA Laboratory (Mercer Island, Wash) using a 20-hour 6 N HCl, 0.05% mercaptoethanol, 0.02% phenol hydrolysis at 115°C. The relative amount of hydroxyproline was calculated using amino acid residue molecular weight and is reported as the μg hydroxyproline/mg tissue. For the sham animals, LV biopsy specimens were obtained from equivalent areas of the LV free wall.

In addition, Masson trichrome staining was performed on histologic sections prepared with a cryostat as described. Collagen content (stained blue) was qualitatively compared between treatment groups.

### MMP Activity

To assess the effects of TIMP-1 transgene overexpression on MMP activity, a separate group of animals underwent injection with Adeno Null (n=4) or Adeno.TIMP-1 (n=4) after ligation of the LAD as described. Animals were euthanized after 2 weeks and border-zone myocardial biopsy specimens were obtained and snap-frozen in liquid nitrogen after removal from the animal. Specimens were pulverized, homogenized in 10 volumes of protein extraction buffer (50 mmol/L Tris, pH 7.5, 1 mmol/L CaCl₂, 0.5% Triton X-100), sheared with a 25-gauge needle, and centrifuged for 1 minute at 6000 rpm to remove cellular debris. The supernatants were
assayed for total protein content (BioRad Protein Assay), and 100 μg of each sample were assayed for MMP activity using the Type I Collagenase Activity Assay Kit (Chemicon) according to the manufacturer’s instructions. Comparison of absorbance to p-aminophenylmercuric acetate-activated MMP-1 control samples (provided by the manufacturer) processed in parallel was performed to quantify the absorbance data. The levels of activity are reported as ng of control MMP activity. It is important to emphasize that control MMP-1 activity is reported only as an indicator of collagen degradation. This does not imply measurement of actual MMP-1 activity in the experimental samples. Instead, we normalized total collagenase activity in the experimental samples to the equivalent activity of the purified MMP-1 provided by the manufacturer.

Statistical Analysis
All values are expressed as mean±SD. The unpaired Student t test was used to calculate the statistical significance between the means of 2 groups. Comparisons between >2 groups were analyzed by ANOVA followed by Tukey-Kramer post hoc testing. P<0.05 was considered to be significant.

Results
Vector Delivery and Transgene Expression
Successful virus delivery and transgene expression was confirmed using a replication-deficient E1, E3-deleted adenovirus containing the beta-galactosidase gene under control of the cytomegalovirus promoter. The viral vector (5×109 pfu in 250 μL) was delivered by direct injection to a rat heart by the methodology described. The rat was euthanized after 1 week, the heart was procured and sectioned, and X-gal staining was performed (Figure 1A). Transmyocardial expression of the transgene, stained blue, was observed throughout the left ventricular free wall area of injection.

Western blotting for human TIMP-1 transgene expression levels 1 week after direct intramyocardial injection into 3 rats revealed high levels of expression in the Adeno.TIMP-1 animals, with no detectable human TIMP-1 protein in the control samples (Figure 1B). It is important to note that the antibody used was raised against human TIMP-1 epitopes; therefore, these results do not suggest that there is no background TIMP-1 expression in native animals, but rather that the level of expression in native animals was below the threshold for cross-detection with the human-derived antibody.

Hemodynamics and Cardiac Function
There was significant preservation of LV systolic function in the Adeno.TIMP-1 group as measured by the maximum LV pressure and the maximum LV dP/dt (Figure 2A,B). In addition, analysis of preload-adjusted maximum power revealed more favorable energetics in the TIMP-1 group (Figure 2C). TIMP-1 overexpression was associated with significant improvement of contractile function, but not normalization to the level of sham uninfarcted animals.

Diastolic function was also significantly preserved in the TIMP-1 animals as measured by the minimum LV dp/dt and tau, the time constant of isovolumic relaxation (Figure 2D,E). Importantly, the TIMP-1 animals had almost complete normalization of diastolic function to the level of the sham group.

Ventricular Geometry and Infarct Size
LV chamber diameter was substantially reduced in the TIMP-1 group compared with the control group (TIMP-1 13.0±0.7 versus control 14.4±0.4 mm, P<0.001; sham 12.2±0.4 mm, P>0.05 versus TIMP-1) (Figure 3A). In addition, the border-zone wall thickness of the TIMP-1 group was significantly greater than that in the control group (TIMP-1 1.59±0.11 versus control 1.28±0.19 mm, P<0.05; sham 1.71±0.23 mm, P>0.05 versus TIMP-1) (Figure 3B). Treatment with Ad.TIMP-1 was therefore associated with a reduction in ventricular dilation and wall thinning compared with control animals and preserved overall ventricular geometry (Figure 3C). There was no significant difference in infarct scar size between the experimental groups (control 45.4±4.8, TIMP-1 48.8±7.5%, P>0.05).

Border-zone Fibrosis
The hydroxyproline content was significantly reduced in the TIMP-1 group compared with control animals (TIMP-1 2.3±0.87 versus control 3.89±1.79 μg hydroxyproline/mg tissue, P<0.05; sham 1.09±0.25 μg/mg, P>0.05 versus TIMP-1) (Figure 4A). Masson trichrome staining was also performed to allow a qualitative evaluation of fibrosis and revealed a reduction of collagen deposition in border-zone areas associated with TIMP-1 treatment. The areas of infarction, in contrast, did not qualitatively differ in collagen content between the 2 groups (Figure 4B).

MMP Activity
To determine the level of MMP activity, we performed an analysis of bovine collagen degradation with a commercially available kit and quantified it in comparison to the activity of an activated form of MMP-1 distributed by the manufacturer. Two weeks after ligation of the LAD and virus injection, the TIMP-1–treated animals had a significant reduction in border-zone MMP activity compared with the control animals (TIMP-1 1.5±0.9 versus control 43.1±14.9 ng of MMP-1 activity, P<0.05) (Figure 5). TIMP-1 overexpression was therefore associated with significant inhibition of MMP activity as determined by our assay.

Discussion
A central component of postinfarction heart failure is ventricular remodeling with global chamber dilatation and wall thinning. We have demonstrated in this study that inhibition of MMPs by adenoviral-mediated myocardial
overexpression of TIMP-1 was associated with significantly improved ventricular geometry after MIs in rats. This reduction in ventricular remodeling was accompanied by a substantial improvement in systolic cardiac function. Interestingly, diastolic function was improved to an even greater degree, and this was associated with a reduction in border-zone fibrosis. Although MMP inhibition was demonstrated, we have not clearly shown a cause-and-effect relationship with this study. However, this is the first report, to the best of our knowledge, of the hemodynamic and remodeling benefits of local myocardial TIMP-1 gene therapy for ischemic cardiomyopathy.

Previous animal studies have highlighted the benefits of systemic pharmacologic inhibition of MMPs in heart failure. However, clinical trials in patients with metastatic cancer have necessitated withdrawal of MMP inhibitor treatment secondary to the development of tendonitis and severe musculoskeletal pain. In addition, MMP inhibition impairs wound healing after injury. Because of these significant limitations, we believe that targeted MMP inhibition in
the myocardium may be a better approach to the treatment of heart failure.

Matrix metalloproteinases play an important role in tissue morphogenesis, wound healing, tumor angiogenesis, metastasis, and atherosclerosis in addition to heart failure. Transgenic TIMP-1/ mice have increased end-diastolic LV volumes, increased end-diastolic LV pressures, and decreased contractility after MI. In addition, chronic myocardial MMP-1 overexpression leads to marked systolic and diastolic cardiac dysfunction in animal models. Transgenic MMP-9 mice have reduced LV remodeling and rupture after MI, suggesting that enhanced post-MI MMP activity is an important contributor to subsequent morbidity and mortality. Reduced TIMP-1 expression and increased MMP activity is also associated with human heart failure. Taken together, these results suggest that an imbalance between MMP expression and TIMP expression is responsible for the increase in proteolytic activity and ECM degradation associated with heart failure.

An elegant study by Heymans et al investigated the effect of metalloproteinase inhibition on the incidence of cardiac rupture. In urokinase-type plasminogen activator and MMP-9 knockout mice, they found that the incidence of cardiac rupture was significantly decreased compared with control animals. However, they also found evidence of impaired infarct healing, decreased angiogenesis, and potentially increased tendency to heart failure 14 days after infarction. They used tail vein adenoviral gene transfer of TIMP-1 and plasminogen activator inhibitor-1 into wild-type animals undergoing infarction and demonstrated a significantly decreased rate of early cardiac rupture, decrease leukocyte infiltration into the infarct, increased areas of

Figure 3. A and B, Left ventricular diameter and border-zone wall thickness 6 weeks after surgery (n=8 per group). Analysis was performed on 2 sections for each animal. C, Histologic sections were stained with hematoxylin and eosin and the areas of diameter (D) and wall thickness (WT) measurement are shown.

Figure 4. A, Hydroxyproline content of border-zone areas adjacent to the infarcts 6 weeks after initial surgery. For the sham group, myocardial biopsy specimens were obtained from equivalent anatomic locations on the ventricular free wall. B, Masson trichrome staining was performed to qualitatively assess the level of collagen deposition, stained blue.

Figure 5. Border-zone MMP activity 2 weeks after injection of either Adeno.Null or Adeno.TIMP-1 vectors in animals undergoing ligation of the LAD (n=4 per group). The collagenase activity was quantified by comparison to a standard curve generated using known amounts of activated MMP-1.
residual necrosis in the infarct, and decreased collagen content in the infarct. Therefore, it appeared that systemically increased levels of TIMP-1 or plasminogen activator inhibitor-1 (an inhibitor of urokinase-type plasminogen activator) resulted in delayed wound healing and decreased scar formation in the infarct. There are several important differences between that study and ours, however. Heymans et al did not report on cardiac function or ventricular geometry in the TIMP-1 treated group, nor did they follow-up the animals long-term to assess development of heart failure. The systemic delivery method for adenoviral vector used in their experiments led to significantly increased serum levels of TIMP-1, which therefore affected not only the central infarct area itself but also all other tissues in the mouse. Importantly, our localized delivery method concentrated TIMP-1 overexpression in the border zone and likely spared the central infarct area. Local MMP activity was not assessed; therefore, a reduction in proteinase activity was not demonstrated in the TIMP-1 overexpression animals. Long-term antifibrotic therapy, however, may actually increase the rate of late aneurysm formation and rupture, and the adenoviral vector strategy we used, with a limited period of overexpression, may actually protect against this.

The myocardial ECM plays an important role in maintaining the structural framework of the heart. Diminished collagen cross-linking as a result of increased MMP activity has been associated with LV dilation and heart failure in animals and humans. Myocyte side-to-side slippage, an important contributor to ischemic ventricular remodeling, may be enhanced by increased MMP activity. We propose that the inhibition of MMP activity observed in the TIMP-1 treated rats in our study resulted in a more stable ECM with preserved collagen fibril structure, cross-linking, and cell adhesion. The result of this enhanced structural stability was diminished LV dilation and improved border-zone wall thickness compared with the control animals.

An interesting finding in this study is that border-zone fibrosis was reduced in TIMP-1 animals as assessed by hydroxyproline content and Masson trichrome staining. Disruption of normal collagen in the infarct and border-zone areas by MMP activity results in enhanced synthesis and deposition of interstitial collagen by myocardial fibroblasts. This newly formed collagen is poorly cross-linked because of ongoing proteolytic activities, and the stimulus for continued deposition remains largely unchecked. In addition, the MMP-mediated degradation of collagen is known to release biologically active peptides that stimulate fibrosis. Reducing MMP activity, therefore, can reduce ongoing fibrosis after an ischemic myocardial insult.

We observed a significant improvement in systolic cardiac function in animals overexpressing TIMP-1. It is likely that this is caused, at least in part, by a reduction in chamber size and improved wall thickness, allowing the heart to work at less mechanical disadvantage. In addition, improved force transduction secondary to a stabilized matrix likely led to further systolic functional benefit. We found, however, that systolic function in the TIMP-1 group was not normalized to the level of sham animals, because improvement was likely limited by the size of the infarction itself and the resultant decrease in functional myocardium. In contrast, diastolic function, as measured by minimum dP/dt and tau, was almost normalized to the level of sham animals with no infarcts. This is likely because of the reduction in myocardial fibrosis, which increases compliance and promotes LV filling. It is important to note, however, that tau is more reflective of the active relaxation of the ventricle and less dependent on the passive stretch properties of the myocardium. It is known that excessive myocardial fibrosis and abnormalities in the ECM result in a significant decrease in myocardial compliance, and thus impairment of diastolic function. It is important to note that diastolic dysfunction is a significant component of ischemic heart failure that is very difficult to manage and treat clinically. Therefore, the improvements in diastolic function conferred by TIMP-1 overexpression are therapeutically very significant findings.

A potential limitation of this study with respect to clinical application is the adenoviral gene delivery method. In human clinical trials, this has been associated with adverse outcomes (including death) and may not be a viable therapeutic treatment modality. However, for the purposes of animal investigations, it provided a reliable and reproducible means to achieve high levels of localized expression in our rodent model. For future clinical applications, further investigations using localized protein delivery vehicles or pharmacologic agents that promote myocardial TIMP-1 expression may be better-tolerated.

The results of the current study lend further support to the important role of MMPs in the development of ischemic heart failure. We have found evidence of enhanced myocardial MMP activity in experimental congestive heart failure, and that this enhanced activity is associated with the development of LV dilation and heart failure. Further, we have found that reducing MMP activity by TIMP-1 overexpression can attenuate deleterious remodeling, reduce myocardial fibrosis, and improve both systolic and, in particular, diastolic cardiac function. Inhibition of metalloproteinase activity by enhanced myocardial TIMP-1 expression is a promising therapeutic target for continued investigation.

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References


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